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Antitumor Effect of *bcl-2* Antisense Phosphorothioate Oligodeoxynucleotides on Human Renal-Cell Carcinoma Cells *in Vitro* and in Mice

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ABSTRACT

***Background and Purpose*: Programmed cell death is a genetically regulated pathway that is altered in many cancers. This process is, in part, regulated by the *bcl-2* oncogene. Antisense oligodeoxynucleotides (ODNs) targeted to specific oncogenes have been used with some therapeutic success in animal models of leukemia and melanoma cells and human Hodgkin's lymphoma. We evaluated the effects of antisense ODNs targeted to the *bcl-2* oncogene on the proliferation of human renal-cell carcinoma (RCC) cells in vitro and on the growth of human RCC xenografts in BALBc nude *(nu/nu)* mice.**

***Materials and Methods*: Expression *bcl-2* mRNA in five RCC cell lines (ACHN, Caki-1, RCZ, RCW, and OS-RC-2) was analyzed by reverse transcriptase–polymerase chain reaction. The effects of phosphorothioated ODNs containing human bcl-2 sense and bcl-2 antisense sequences that were transfected with Lipofectin on the proliferation and viability of cultures of established human RCC cell lines were determined by MTS assay. The expression of Bcl-2 protein in ACHN tumor cells following antisense bcl-2 (AS2) ODN treatment was evaluated by Western blot analysis, and the extent of apoptosis in these cells was determined by fluorescence-activated cell sorter (FACS) analysis. The antitumor activity in ACHN xenografts in *nu/nu* mice was monitored by measuring differences in tumor weight in treated and control mice.**

***Results*: Expression of bcl-2 mRNA was detected in all five RCC lines. Treatment with antisense bcl-2 ODNs inhibited the growth of all tested RCC cells and decreased Bcl-2 protein expression in ACHN cells. The AS2 antisense ODN complementary to the coding region of *bcl-2* mRNA showed a superior antiproliferative effect compared with AS1 ODN complementary to the translation initiation region. Inhibition by antisense bcl-2 ODNs of ACHN cells was dose dependent. The FACS analysis revealed that growth inhibition was associated with the induction of programmed cell death. *In vivo*, AS2 ODN antitumor activity was noted in locally injected groups.**

***Conclusions*: Treatment of human RCC with antisense ODNs targeted to *bcl-2* inhibits growth and is associated with the induction of programmed cell death. These results suggest therapeutic use of antisense bcl-2 in the treatment of RCC.**

INTRODUCTION

APPROXIMATELY 30% OF PATIENTS with renal-cell carcinoma (RCC) present with metastatic disease.[1] In addition, a subset of patients with localized tumors ultimately develop metastatic disease, and some patients are at risk for local tumor spread or progression. To date, cytotoxic chemotherapy agents have not improved the survival of patients with RCC with metastasis. A more rational strategy for improving survival is needed.

Cancer development is a complex, multistep process characterized by the abnormal activation of cellular proto-oncogenes (to become oncogenes) and the loss of function of tumor suppressor genes.[2] Individual oncogenes, encoding molecules involved in the transduction of mitogenic signals or in regulat-

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ing the transcription of genes whose products are normally required for DNA synthesis and cell proliferation, have been implicated in this process.

The *bcl-2* oncogene was first identified as a site involved in a t(14;18) chromosomal translocation in follicular lymphomas.[3] The *bcl-2* gene is the prototype of a novel class of oncogenes that contribute to neoplastic progression by enhancing tumor cell survival through inhibition of apoptotic death. The exact molecular mechanism by which Bcl-2 inhibits programmed cell death has not been unequivocally established. Studies have shown that Bcl-2 inactivates inducers of programmed cell death, such as Bax, through heterodimerization and that it is the ratio of Bcl-2, or functionally similar gene products, to apoptosis inducers that determines whether a cell will undergo programmed death.[4] The antitumor activity of radiation and many chemotherapeutic agents appears to result from apoptosis that is dependent on the expression of Bcl-2 in a variety of animal tumor models and non-Hodgkin's lymphoma.[5,6] Thus, *bcl-2* appears to be a particularly attractive target for genetic downregulation in a wide range of tumor types, especially in concert with conventional cytotoxic agents.[7]

Antisense oligodeoxynucleotides (ODNs) can inhibit gene expression and are now commonly used to investigate the role of oncogenes in cancer development.[8–10] In mice with human leukemia cell transplants, systemic treatment using antisense ODNs temporarily curbed disease progression.[11] Antisense ODNs targeting several oncogenes have recently been reported to specifically inhibit expression of these genes and delay tumor progression. The therapeutic potential of antisense ODNs in the treatment of solid tumors also has been investigated.[11–13] Recently, antisense bcl-2 ODNs were found to inhibit the growth of small-cell lung cancer and melanoma *in vitro*.[14–16] Webb and colleagues[17] treated relapsed non-Hodgkin's lymphoma with antisense bcl-2 ODNs given subcutaneously. Four of the nine patients showed a complete response or stable disease. Antisense ODNs targeted to activated oncogenes also might have a therapeutic role in the treatment of human malignancies, both *ex vivo* and *in vivo*, and have been used with some therapeutic success in animal models of neoplastic disorders.

The present study represents the first in a series of preliminary experiments leading up to attempted gene therapy for RCC employing antisense bcl-2 ODNs. Herein, we establish that bcl-2 are expressed in five RCC cell lines and then demonstrate the programmed cell death-inducing antiproliferative effect of antisense bcl-2 ODNs on the ACHN cell line *in vitro* and in human RCC xenografts growing in nude mice.

MATERIALS AND METHODS

Cell Lines

Four kinds of human RCC cell lines (ACHN, Caki-1, RCZ, and RCW) were obtained from the American Type Culture Collection (Rockville, MD). The OS-RC-2 cell line was a kind gift from Dr. T. Kinouchi. Cells were maintained in Eagle Minimal Essential Medium (MEM) or RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (ICN Biomedicals Inc., Aurora, OH), penicillin 100 units/mL, streptomycin 100 μg/mL, and 4 mM glutamine at 37°C in an atmosphere of 5% CO_2. Cells were plated and harvested in either the log phase or confluency for all experiments.

Reverse transcription–polymerase chain reaction analysis of bcl-2 mRNA

The RT-PCR procedure involved isolation of total mRNA using the ISOGEN RNA isolation kit (ISOGEN, Toyama, Japan) from cells in log phase or confluent growth. The RT-PCR was performed using a Takara RNA LA PCR kit v. 1.1 from Takara Shuzo (Otsu, Japan). First-strand cDNA was synthesized using 1.5 μg of total RNA template reverse transcribed using recombinant RT. Random hexamers (50 pM) were used in a 20-μL reaction mixture containing 5 mM $MgCl_2$, 1× RNA PCR buffer, 10 mM dNTP mix, and 20 U of RNase inhibitor. The reaction mixtures were incubated at 30°C for 10 min and at 55°C for 15 min, then heated for 5 min at 99°C. The reaction mixture was then diluted to a final volume of 40 μL with sterile H_2O. The PCR mixtures contained 5 μL of diluted RT-PCR product, 1.5 mM $MgCl_2$, 1× RNA PCR buffer, 10 mM dNTP mix, 20 pM forward and reverse primers of bcl-2 and β-actin using ApoPrimer set (Takara Shuzo) and 2.5 U of *Taq* polymerase (Takara Shuzo Co.) in a final volume of 50 μL. The DNA thermal cycler (ASTEC, Fukuoka, Japan) was run at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min for 35 cycles. The PCR products were electrophoresed on a 3% agarose gel in 1× TBE buffer. Experiments were done in duplicate.

Oligodeoxynucleotides

Two kinds of bcl-2 antisense ODNs—AS1, complementary to the translation initiation (5'-TCTCCCAGCGTGCGCC-AT-3'), and AS2, complementary to the coding region (5'-AATCCTCCCCCAGTTCACCC-3') of *bcl-2* mRNA—were used.[14,17] As controls, S1 (5'-TACCGCGTGCGACCCTCT-3') and S2 (5'-CCCACTTGACCCCCTCCTAA-3') sense bcl-2 ODNs were used. All ODNs were phosphorothioated, purified, and dried under vacuum by Espec Oligo Inc. (Tsukuba, Japan). The ODNs were resuspended in serum-free OPTI-MEM (Life Technologies, Ltd., Gaithersburg, MD, USA) for use in *in vitro* and *in vivo* analysis.

DNA Transfections

Transient transfections were performed using Lipofectin reagent (Life Technologies), which is a 1:1 (w/w) liposome formulation of the cationic lipid N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and dioleoyl phosphotidylethanolamine (DOPE) in membrane-filtered water. Antisense and sense bcl-2 ODNs were incubated for 15 min with Lipofection 10 μg/mL in serum-free OPTI-MEM at room temperature. All medium containing Lipofectin was prepared just before each experiment.

Detection of Transfection Efficiency of Lipofectin in RCC Cells

In situ staining of cells for β-galactosidase activity was performed to identify the transfection efficiency of Lipofectin in RCC cells. Briefly, each cell preparation was cultured with 0.5 μg of β-galactosidase control plasmid (Promega, Madison, WI) in Lipofectin 0, 2.5, 5, and 10 μg/mL in a 60-mm culture dish.

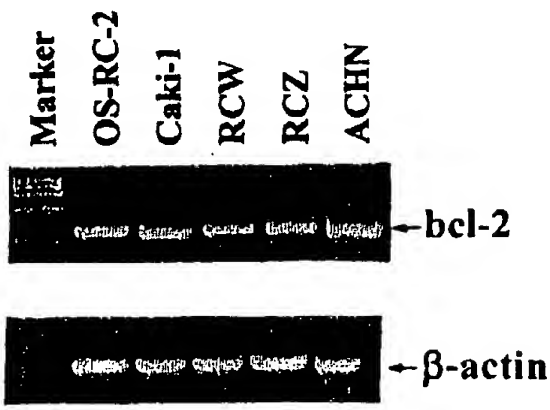

FIG. 1. Expression of *bcl-2* mRNA in RCC cell lines. mRNA was isolated from cells in log phase or confluency, and RT-PCR was performed for first-strand cDNA synthesis and amplification. Experiments were performed in duplicate. Equivalence of total RNA amounts in samples was assessed by comparison of β-actin RNA levels.

Cells were washed with 1× phosphate-buffered saline (PBS), fixed by 0.5% glutaraldehyde solution, and incubated for 15 min. Cells were rinsed gently three times with 1× PBS to remove the glutaraldehyde. Filtered 0.1% X-gal solution (0.2% X-gal, 2 mM $MgCl_2$, 5 mM $K_4Fe[CN]_6 \cdot 3H_2O$, 5 mM $K_3Fe[CN]_6$) (Promega, Madison, WI) was added, and the mixtures were incubated for 11 h. Stained cells were viewed with a light microscope.

In Vitro *Cell Growth Assay*

The growth-inhibitory effects on ACHN, RCW, and OS-RC-2 RCC cells were assessed daily on the basis of cell number and viability by the CellTiter 96™ Aqueous nonradioactive one-solution cell-proliferation assay kit (Promega), which contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-^{2}H-tetrazolium, inner salt; MTS] and an electron-coupling reagent (phenazine ethosulfate; PES). For the CellTiter 96 Aqueous Assay (Promega), 8×10^3 cells of each line were seeded in 96-well microtiter plates and allowed to attach overnight. The next day, the medium was replaced with 10% FBS-conditioned MEM or RPMI 1640 containing various doses of ODNs and controls with or without Lipofectin. Every 24 h of incubation, 20 μL of CellTiter 96 Aqueous reagent was added to each well, followed by incubation for 4 h at 37°C. The optical density was determined with a microculture plate reader (Flow Laboratories, Lugano, Switzerland) at 492 nm. Absorbance values were normalized to the values obtained for the vehicle-treated cells to determine percent survival. Each assay was performed in triplicate.

Detection of Bcl-2 Protein by Western Blot Analysis

The expression of Bcl-2 protein in the ACHN cells was examined by Western blot analysis. The cells were treated with 150 nM each AS1, S1, AS2, and S2 ODNs with Lipofectin 10 μg/mL. Untreated cells and cells treated with Lipofectin without ODNs were used as controls. At 48 h, cultured cells were placed in lysis buffer (0.01M Tris HCl, pH 7.4; 0.15 M NaCl, 5 mM EDTA, 1% Triton X-100, pepstatin 0.7 μg/mL, aprotinin 0.23 U/mL, 10 mM leupeptin, 1 mM benzamide, and 1 mM phenylmethylsulfonyl fluoride) and sonicated. The protein content of the samples was quantified using the BCA protein assay (Pierce Chemical Co., Rockford, IL). Protein extracts (20 μg) from lysates of the cultured cells were electrophoresed on a 12% SDS–polyacrylamide gel and transferred to a nitrocellulose filter (Millipore, Tokyo, Japan). The filters were blocked at 4°C overnight in PBS containing 10% nonfat milk powder, and then incubated for 1 h with 1:1000 mouse anti-human Bcl-2 monoclonal antibody (clone 124; DAKO A/S, Glostrup, Denmark) or 1:5000 anti-human β-actin monoclonal antibody (clone AC-74; Sigma Chemical Co.). To detect the primary antibody, we incubated blots with anti-mouse IgG horseradish peroxidase conjugate (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. The immunocomplex was visualized by enhanced chemiluminescence by use of the ECL detection kit (Amersham Life Science, Buckinghamshire, England) according to the recommendation of the manufacturer, followed by exposure to X-ray film (Amersham Life Science). The relative protein level was quantified after the films had been scanned with a flat bed scanner (Shimadzu, Kyoto, Japan).

Cell Cycle Analysis of Antisense bcl-2-Treated ACHN Cells

Expression of Bcl-2 was also evaluated in ACHN cells by fluorescence-activated cell sorter (FACS) analysis following *in vivo* administration of bcl-2 ODNs. The ACHN cultures, growing in six-well plates, were exposed to bcl-2 ODNs and control materials as described above. Cells were harvested at 48 h, washed with PBS, and fixed at 4°C for at least 30 min with a solution of 80% ethanol in PBS. Samples containing 2×10^6 fixed cells were stained with a solution containing RNase A75 KU/mL and propidium iodide (PI) 50 μg/mL (Sigma) and were analyzed using FACScan (Becton Dickinson, San Jose, CA). Red PI fluorescence was monitored using an LP 620 filter. Twenty thousand events per sample were stored, and the histograms were analyzed further using Cell Quest v. 3.1 (Becton

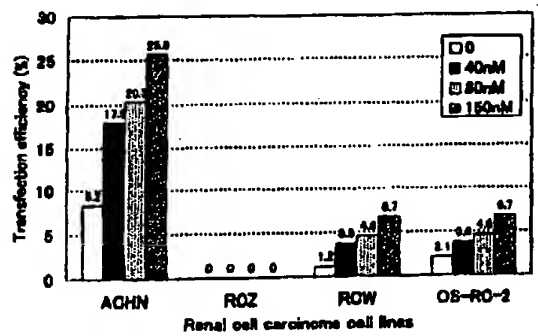

FIG. 2. Transfection efficiency of Lipofectin in RCC cells. Transfection efficiency with β-galactosidase control plasmid 0.5 μg/mL in Lipofectin 0, 2.5, 5, and 10 μg/mL was identified in ACHN, RCZ, RCW, and OS-RC-2 cells.

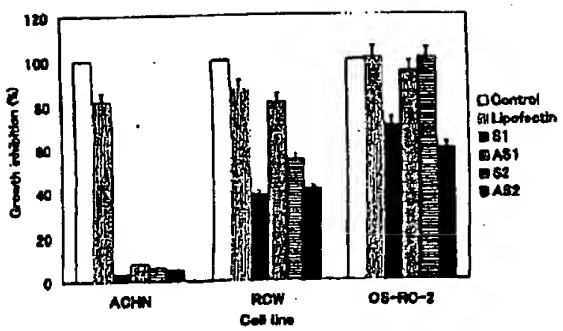

FIG. 3. Growth inhibitory effects on ACHN, RCW, and OS-RC-2 cells of antisense bcl-2 (AS1 and AS2) and sense bcl-2 (S1 and S2) ODNs and control with or without Lipofectin for 4 days beginning 1 day after cell seeding. Cells were treated with 150 nM ODNs on days 2 through 4. Each point is mean ± SD of triplicate samples. All experiments were repeated at least once.

Dickinson) to estimate the percentage of cells in various phases of the cell cycle.

In Vivo *Administration of Antisense bcl-2 ODNs*

A series of BALBc *nu/nu* mice, 6 to 8 weeks old and 22 to 25 g in body weight, were purchased from CLEA Japan, Inc. (Tokyo) and housed in a pathogen-free environment at 22°C on a 12-h light, 12-h dark cycle. Mice were given acidified, autoclaved water and an X-irradiated commercial diet. All manipulations were performed under sterile conditions in a laminar flow hood. Each experimental group of mice included at least six animals.

For *in vivo* study, approximately 1×10^7 cells of the ACHN line suspended in 200 μL of Matrigel (Collaborative Biomedical Products, Bedford, MA) were injected subcutaneously into the lower right flank. When the implanted tumor had grown to about a 200-mg volume, 1 mg of antisense bcl-2 (AS2) with or without 1 mg of Lipofectin dissolved in 100 μL of serum-free OPTI-MEM was injected locally into each tumor three times a week for 4 weeks. Untreated and Lipofectin-only groups were used as controls. The growth of the subcutaneous tumors was measured serially with calipers. The greatest length of a tumor mass (a) and the width perpendicular to it (b) were measured every 7 days, and the tumor size was reported as $a^2 \times b/2$. Toxicity was assessed on the basis of apparent drug-related deaths and net body weight loss. Net body weight loss was calculated as a percentage of the mean net body weight of untreated mice.

Statistical Analysis

All statistical analyses were performed using commercial software (Statview 5.0; Abacus Concepts Inc., Berkeley, CA, USA). The significance of the difference between two observations was determined using a two-tailed Student's *t*-test. *P* values <0.05 were considered to reflect statistically significant differences. Linear regression analysis was performed on *in vivo* tumor growth curves, and the slopes were compared to evaluate the statistical significance of the differences.

RESULTS

Expression of bcl-2 mRNA in RCC Lines

To establish whether the antitumor effect observed *in vitro* was attributable to a specific antisense effect, bcl-2 mRNA expression in each cell line was evaluated by RT-PCR. Expression of bcl-2 mRNA was readily detected in all five RCC cell lines (Fig. 1). Variations in template cDNA concentrations were normalized by coamplification of β-actin.

Transfection Efficiency of Lipofectin in RCC

Dose-dependent transfection efficiency was noted in ACHN, RCZ, RCW, and OS-RC-2 cells. In ACHN, transfection efficiency with a β-galactosidase control plasmid 0.5 μg/mL in Lipofectin 0, 2.5, 5, and 10 μg/mL was 8.2%, 17.9%, 20.3%, and 25.6%, respectively. In RCW cells, transfection efficiency under the same conditions was 1.2%, 3.8%, 4.6%, and 6.7%, respectively. In OS-RC-2 cells, the transfection efficiency was 2.1%, 3.8%, 4.6%, and 6.7%, respectively. No transfection was identified in RCZ cells (Fig. 2).

In Vitro *Growth Assay and Western Blot Analysis*

The most growth inhibitory effect of treatment with 150 nM AS1, S1, AS2, and S2 ODNs in Lipofectin was noted in ACHN cells compared with RCW and OS-RC-2 cells. The AS2-treated groups showed the most growth inhibitory effect in all three RCC lines. No inhibitory effect was found in untreated and Lipofectin-only cells. Interestingly, S1 and S2 treatment inhibited growth in ACHN and RCW cells (Fig. 3). To examine the growth inhibitory effect of various concentrations of ODNs, 80 nM and 150 nM concentrations of each ODN with Lipofectin were added to ACHN cells. All groups except the control and Lipofectin-only showed growth inhibition. All 150 nM treatment groups showed a greater growth inhibitory effect than 80 nM groups (Fig. 4).

The effect of treatment at 150 nM AS1, S1, AS2, and S2 with Lipofectin on Bcl-2 protein expression in ACHN cells at 48 h was evaluated by Western blot analysis. Untreated control

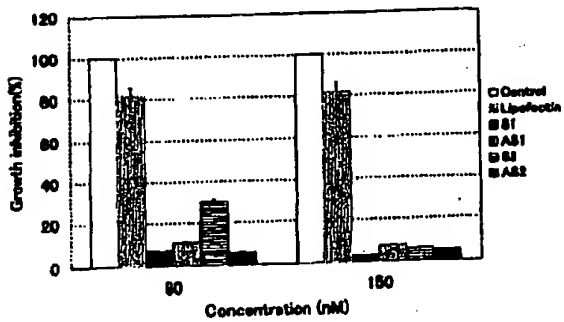

FIG. 4. Dose-dependent effect of bcl-2 ODNs and control in ACHN cells. Cells were treated with 80 nM and 150 nM ODNs with Lipofectin. Each point is mean ± SD of triplicate samples. All experiments were repeated at least once.

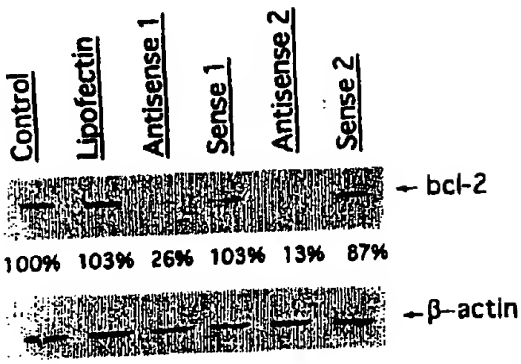

FIG. 5. Representative Western blot analysis of Bcl-2 expression in ACHN cells exposed to 150 nM bcl-2 ODNs. Analysis was performed at 48 h of ODN treatment with untreated control, Lipofectin, AS1, S1, AS2, and S2. Expression of β-actin served as control for uniformity of protein gel loading and blotting. Bcl-2 and β-actin proteins were detected simultaneously by chemiluminescence. Experiment was repeated twice.

and Lipofectin without ODNs cultures were used as controls. The Bcl-2 protein expression decreased in cells treated with AS1 (26%) and AS2 (13%). However, no suppression was noted in the untreated control, Lipofectin, S1, and S2 groups (Fig. 5). Densitometric evaluation confirmed that Bcl-2 levels were normalized to β-actin levels.

FACS Analysis of Apoptosis after In Vivo bcl-2 Antisense ODNs Treatment

Analysis of the DNA content histograms by FACS analysis revealed an accumulation in the hypodiploid region representative of a population undergoing apoptotic cell death. A greater percentage of the ACHN cells derived from AS2-treated cells exhibited the characteristics of apoptosis. The bcl-2 antisense ODNs induced apoptosis at 48 h in 32.1% and 43.2% of the ACHN cells treated with AS1 and AS2 + Lipofectin, respectively; these cells showed a hypodiploid DNA content, indicative of apoptosis. In contrast, untreated control, cells treated with Lipofectin alone, and cells receiving S1 and S2 with Lipofectin showed 5.7%, 6.6%, 5.9%, and 11.4% hypodiploid DNA contents, respectively (Fig. 6).

In Vivo *Antitumor Effect of bcl-2 [S]ODN Treatment on ACHN RCC Line*

Nude mice bearing ACHN tumors were treated with 1 mg of AS2/mouse three times a week for 4 weeks (total 12 mg). Groups having intratumor injections of AS2 with Lipofectin showed a statistically significant reduction of tumor weight ($P < 0.05$–0.01) at 2 to 6 weeks after treatment. However, AS2 without Lipofectin, control with Lipofectin, and untreated control groups did not show any reduction (Fig. 7). The maximum effect on tumor growth was evident at 2 to 3 weeks after treatment. No death or body weight loss was observed (data not shown).

DISCUSSION

The Bcl-2 protein has a unique ability to block cell death. The *bcl-2* gene product regulates programmed cell death, and a number of studies have suggested that *bcl-2* is involved in the selection and maintenance of long-living cells and in rescuing them from apoptotic death.[4–6,18,19] As cancer is a disease of altered cellular homeostasis, recent strategies have targeted the induction of programmed cell death as an attractive paradigm for the treatment of this disease.[20]

Several studies have demonstrated high levels of Bcl-2 expression in cancers of the colorectum, stomach, breast, ovary, and prostate, as well as neuroblastoma and acute and chronic leukemias.[7,21] In colorectal cancers, Bcl-2 expression may represent an early event in tumorigenesis, as cells in the parabasal and superficial regions of dysplastic polyps are more likely to display Bcl-2 staining.[22]

In RCCs, Bcl-2 protein expression has been detected in virtually all clear-cell carcinomas, around 60% to 80% by immunohistochemical analysis.[25–26] However, Bcl-2 positivity was not associated with any pathological features. A Bcl-2-positive reaction was somewhat associated with a poor prognosis compared with Bcl-2-negative cases, although a statistical difference between the two groups was not observed. Vasavade

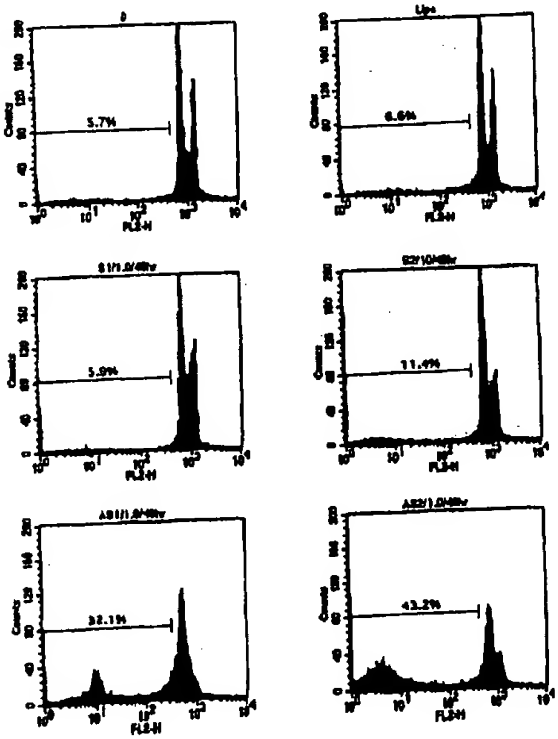

FIG. 6. Effect of bcl-2 ODNs and control on induction of apoptosis as shown by FACScan analysis of DNA content at 48 h. Percentages indicate proportion of cell population with subdiploid DNA content. Experiment was repeated twice.

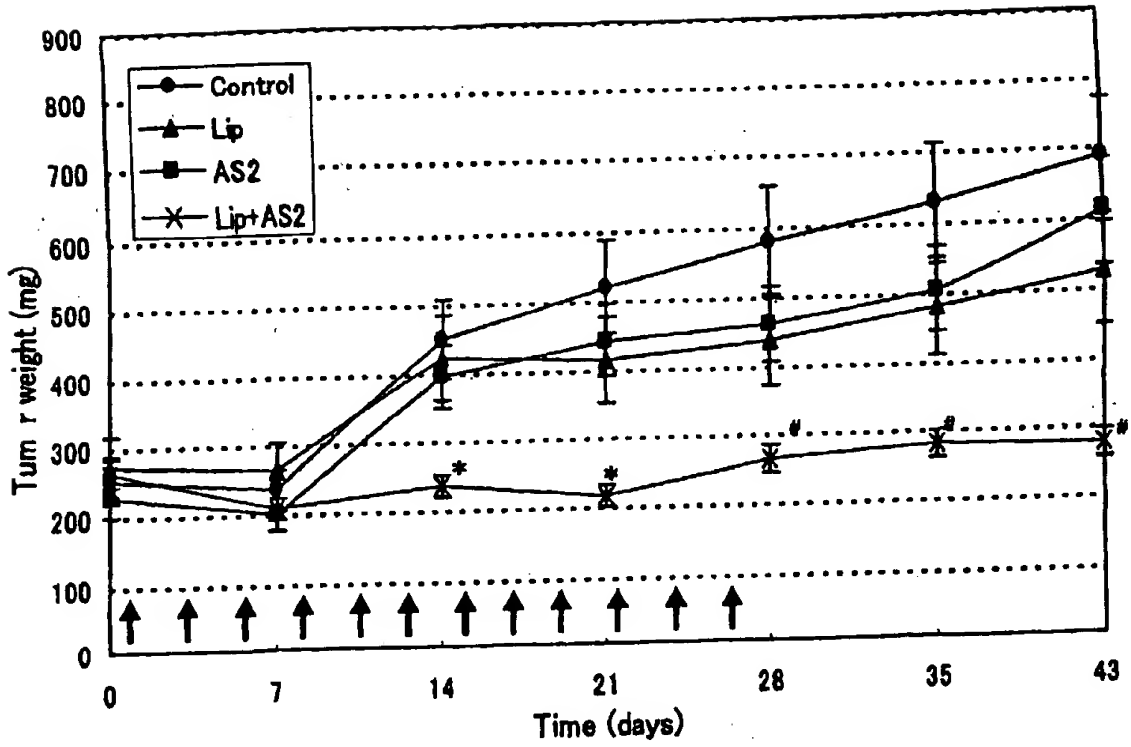

FIG. 7. Effect of AS2 antisense bcl-2 ODNs on growth of ACHN tumor implanted in BALBc nu/nu mice. When implanted tumor was grown to about 200-mg volume, each treatment was started. Each point is mean ± SD. Arrows indicate injection of AS2 ODNs.

and associates[24] reported that a high level of Bcl-2 staining, especially in the clear-cell subtype of RCC, was identified in 80% of cases. This expression pattern correlated well with higher tumor grade, but alone as a predictor of adverse outcome, it did not achieve a statistical significance.[24] Lipponen and coworkers[27] reported that Bcl-2 was expressed in 20% of tumors, being particularly common in well-differentiated small tumors and low-stage tumors ($P < 0.0001$). In addition, infrequent expression of Bcl-2 in RCC was reported, with only 2 of 31 cases (7%) showing positive staining.[28] In our study, 51 of 113 RCCs (45%) showed Bcl-2 expression, but there was no correlation with tumor grade or stage and prognosis by immunohistochemical analysis.[29] Perhaps higher degrees of Bcl-2 staining reflect a higher propensity for cell cycle misregulation.

Therapeutic strategies for those diseases overexpressing Bcl-2 have focused on decreasing Bcl-2 levels. Antisense ODNs are chemically modified stretches of single-strand DNA that are complementary to mRNA regions of a target gene. Antisense ODNs can inhibit gene expression by forming RNA:DNA duplexes, thereby reducing the activity of the target gene products.[30] Recent reports have shown that antisense bcl-2 ODNs induce apoptosis in various types of malignant cell lines *in vitro*, including small-cell lung cancer, myeloma, leukemia, lymphoma, and cholangiocarcinoma.[5,8,8-16,31-35] Furthermore, combined use of antisense bcl-2 ODNs and chemotherapeutic agents resulted in a synergistic inhibition of small-cell lung cancer cells *in vitro* and melanoma cells both *in vitro* and in vivo.[4,14] In fact, antisense bcl-2 ODNs therapy has recently been conducted in patients with non-Hodgkin's lymphoma, resulting in some initial success.[17]

Our data have shown that *bcl-2* mRNA is expressed in all of five RCC lines and that bcl-2 antisense ODNs are effective inhibitors of RCC growth *in vitro* as well as *in vivo*. The bcl-2 antisense ODNs (AS1 and AS2) revealed different levels of inhibition of growth of ACHN cells. Ziegler and colleagues[14] examined the inhibitory effect of 13 antisense ODNs targeting various regions of the *bcl-2* mRNA sequence to identify the most effective sequence(s) for reducing Bcl-2 protein level in small-cell lung cancer. Their ODN 2009 that targeted the coding region of the *bcl-2* mRNA was the most cytotoxic. In our study, AS2 (same as 2009) showed a greater antiproliferative effect than AS1, which targets the translation initiation sites of the *bcl-2* mRNA used for treating relapsed non-Hodgkin's lymphoma.[17]

The therapeutic index of bcl-2 antisense preparations was closely related to the dose. The dose–response relation and the absence of antitumor effects with the sense bcl-2 ODNs controls support the hypothesis that bcl-2 antisense ODNs are responsible for the antitumor activity observed. However, the most convincing demonstration of bcl-2 antisense ODNs sequence specificity is the downregulation of Bcl-2 protein levels in ACHN cells *in vitro*.

Leonetti and associates[36] reported that a total ODN dose of

8 mg/mouse given in a single cycle of treatment for 8 consecutive days (1 mg/mouse per day) was highly toxic, resulting in a 50% death rate. Moreover, 1 mg/mouse per day of sense and scrambled ODNs given on the same schedule also caused toxic effects in the absence of antitumor activity. In our *in vitro* study, S1 and S2 sense ODNs showed growth inhibition but not reduced Bcl-2 protein expression compared with the antisense AS1 and AS2. Yanwen et al[37] reported that phosphorothioated ODNs, including the guanine quartet sequence, showed a nonspecific antiproliferative effect. In addition, ODNs containing excellent matches for the CpG motif cause lymphocytic activation *in vitro* and *in vivo*.[38] These data suggest that nonspecific toxicity might reflect some physicochemical property of phosphorothioated compounds. In our *in vivo* study, no adverse effect was noted in mice receiving a total dose of 12 mg of antisense bcl-2 ODN/mouse for 4 weeks. Additional studies are required to assess the effects of bcl-2 antisense phosphorothioated ODNs on normal cells *in vivo*.

The antitumor effects of bcl-2 antisense ODNs have been obtained at relatively high concentrations. At the beginning of gene therapy using antisense bcl-2 ODNs *in vitro*, effective concentrations of ODNs were about 50 to 250 μM.[39] Then, phosphorothioated ODNs have been developed and used at 0.3 to 10 μM concentrations.[31,40,41] Phosphorothioated ODNs are stabilized to resist nuclease digestion because one of the nonbridging phosphoryl oxygens of DNA has been replaced with a sulfur. Recently, Ziegler and coworkers[14] used 0.15 μM phosphorothioated antisense bcl-2 ODNs with liposome transfection using N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP). In addition, Gleave et al[42] reported that 500 nM antisense bcl-2 ODNs with Lipofectin inhibited cell growth. In our study, the transfection efficiency increased in a dose-dependent manner from 8.2% without Lipofectin to 25.6% with Lipofectin 10 μg/mL, and 150 nM antisense bcl-2 ODNs showed an antiproliferative effect in RCC cells. The effective concentration of antisense ODNs has been decreased. However, further development is needed to overcome many difficulties, among which are high cost and difficulties in the large-scale production of ODNs. We hope that the development of third-generation ODN analogs or more efficient ODN delivery will lead to therapeutic applications in humans.

CONCLUSION

The present study confirms that inhibition of bcl-2 function by administration of antisense bcl-2 ODNs interferes with established growth of RCC cell lines *in vitro* and *in vivo*. We showed that Bcl-2 protein levels decrease after antisense bcl-2 treatment, and the decrease in cellular proliferation is secondary to programmed death. However, because numerous genes mediate tumor progression, inhibition of a single target gene is unlikely to be sufficient to suppress tumor progression completely. Indeed, there has been only one report demonstrating a case of complete response in relapsed non-Hodgkin's lymphoma after treatment with antisense bcl-2 ODN.[17] Accordingly, use of antisense ODNs targeting anti-apoptotic molecules may enhance the extent of apoptosis-initiated chemotherapeutic or hormonal agents, improving control over tumor progression.

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